Neglected aspects of hormone mediated maternal effects

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Chapter 4

Avian yolk androgens are metabolized rather than taken up by the embryo during the first days of incubation

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ABSTRACT

Several studies show effects of yolk androgens in avian eggs on the phenotype of the offspring. Yolk hormone concentrations decline strongly within the first few days of incubation. Although early embryonic uptake of yolk androgens is suggested by the presence of radioactivity in the embryo when eggs are injected with radiolabelled androgens, these studies do not verify the chemical identity of radioactive compound(s), although it is known that these androgens can be metabolized substantially. By using stable isotope-labelled testosterone and androstenedione in combination with mass spectrometry, enabling verification of the exact molecular identity of labelled compounds in the embryo, we found that after 5 days of incubation the androgens were not taken up by the embryo. However, their concentrations in the entire yolk albumen homogenates declined strongly, even when corrected for dilution by albumen and water. Our results indicate metabolism of maternal androgens, very likely to 5β-androstane-3α,17β-diol, etiocholanolone and their conjugated forms. The results imply that the effects of increased exposure of the embryo to maternal androgens take place either before this early conversion or are mediated by these metabolites with an as yet unknown function, opening new avenues for understanding hormone mediated maternal effects in vertebrates.

1 INTRODUCTION

Exposure of the vertebrate embryo to maternal hormones can have long-lasting effects on offspring morphology, physiology, and behaviour in a wide array of taxa, including fish (Brown et al. 2014; Guiguen et al. 2010; Pri-Tal et al. 2011), reptiles (Clairardin et al. 2013; Paitz & Bowden 2011; Radder 2007), birds (Gil 2008; Schwabl 1993; von Engelhardt & Groothuis 2011), and mammals (Del Giudice 2012; Drea 2011; Harris & Seckl 2011). In egg-laying vertebrate species, such as birds, almost the entirety of embryonic development occurs outside the mother's body, facilitating measurement and manipulation of this prenatal hormonal exposure. Steroid hormone deposition by the avian mother into her eggs varies with environmental cues (Eising et al. 2001; Gil 2008; Hahn 2011; Müller et al. 2002; Schwabl 1993, 1997a; von Engelhardt & Groothuis 2011; Welty et al. 2012). Hormone manipulation of avian eggs revealed a wide array of phenotypic effects on the offspring, including traits such as hatching time, hatching success, metabolic rate, immune function, endocrine function, growth, competitiveness, reproduction, mate choice, and survival rate (von Engelhardt & Groothuis 2011). It is generally thought that these hormone mediated maternal effects are adaptive, providing a maternal "weather forecast" for the chick to adjust its phenotype to the environment that it will experience after hatching (Groothuis et al. 2005b). However, despite the many descriptive and functional studies in this field, underlying mechanisms of how and when the embryo is able to react to the maternal hormones are still not known. This hampers progress in the research field (Carere & Balthazart 2007; Groothuis & Schwabl 2008), for example, in understanding the role of the offspring in family conflict (Müller et al. 2007).

In order to be functional, these maternal egg hormones must reach the offspring's tissues, but whether this is indeed the case is as not yet clear. Several studies indicate a strong decrease of yolk hormone concentrations already within the first few days of egg incubation (Bowden et al. 2002b; Eising et al. 2003; Elf & Fivizzani 2002; Feist & Schreck 1996; Paitz & Bowden 2009; Wilson & McNabb 1997) and one study showed that this is not due to mixing of yolk with albumen (Paitz et al. 2011), raising the question as to whether this is due to embryonic uptake or metabolism, and to what extent and which metabolites really reach the embryo. One explanation for the decrease in androgens and estrogens during early incubation is that conjugation of active steroids takes place facilitating uptake of these hormones from the lipophilic environment (the yolk), after which the embryo may deconjugate the hormone when and where it is required (Paitz & Bowden 2008, 2013).

Additional complexity is added by the fact that a steroid of interest found in embryonic tissue might not be of maternal origin but could be synthesized *de novo* by the embryo itself. Such *de novo* synthesis may be due to the activity of maternal or embryonic enzymes in the egg using maternal steroids and/or cholesterol in the yolk as a substrate (Bruggeman et al. 2002; Nomura et al. 1999). Therefore, any steroids detected in the embryonic tissues

even in early development could have been derived *de novo* from precursors. In order to resolve this problem, radioactive steroids have been injected into egg yolk, followed by detection of radioactivity in the embryonic tissues (Benowitz-Fredericks & Hodge 2013; von Engelhardt et al. 2009). However, these studies could only detect radioactivity originating from the labelled hormones in embryonic tissues and did not verify the molecular identity of the radio-labelled compound. A few other studies analysing steroid metabolism in incubated eggs suggested that maternal steroids were metabolized and conjugated (Paitz et al. 2011, 2012; Vassallo et al. 2014). This indicates that radioactive compound(s) in the embryonic tissue could have been a different metabolite, or even a biologically inactive form of it, rather than the originally injected steroid.

In order to study early hormone metabolism and embryonic uptake, and to differentiate maternal from other newly synthesized compounds, we injected a stable isotope labelled form of the most frequently studied steroid in this field, testosterone ([$^{2}H_{3}$]T), and its precursor androstenedione ([$^{13}C_{3}$]A4), into chicken egg yolk, and incubated the eggs for either zero or five days. The advantage of using stable isotope labels is that the metabolic outcome can be tracked using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS), a method that is very accurate for molecular identification and can distinguish between naturally occurring and the heavy isotope labelled compounds that differ only with respect to their mass. The incubation time of five days was chosen because the gonadal differentiation (Smith et al. 1997; Yoshida et al. 1996) and the surge of the endogenous steroid production (Woods et al. 1975) in chicken embryos starts only after five days of egg incubation. The injected hormone values were within two standard deviations of the mean yolk hormone concentrations (T = 0.74 ± 0.13 pg/mg; A4 = 23.24 ± 2.20 pg/mg; means ± s.d.) obtained by mass spectrometry in unincubated eggs (day 0) laid on the same day.

We characterized the *in ovo* dynamics of the labelled T and A4 over the first five days both in the yolk-albumen homogenate and in the embryo, separating the embryo itself and its extra-embryonic membranes, including the yolk sac membrane where the uptake of yolk and its hormones to the circulation of the embryo is likely to take place. The chicken embryo expresses the steroidogenic genes P-450scc, 3β-HSD, P-450c17 and 17β-HSD, but not aromatase (Nomura et al. 1999) before day 5, which is necessary to convert androgens into estrogens, so we only investigated the androgen pathways. The majority of maternal hormones are deposited in the yolk, but the yolk becomes mixed with albumen early during incubation. Therefore, the decline in yolk hormone concentrations reported in the literature (Bowden et al. 2002b; Eising et al. 2003; Elf & Fivizzani 2002; Feist & Schreck 1996; Paitz & Bowden 2009; Wilson & McNabb 1997) might be due not to metabolism but rather to the dilution of yolk, for which no evidence was found in European starling (Paitz et al. 2011) and rock pigeon (Kumar et al. 2018b) eggs, but is as yet unknown for chicken eggs. Therefore, we performed the study in the yolk-albumen homogenate, enabling us to distinguish

between both hypotheses. The embryonic tissue and its extra-embryonic membranes were analysed separately.

2 MATERIALS AND METHODS

2.1 Animal ethics

This study used 5-day-old chicken embryos, and thus does not require an ethical license or approval from an animal experimentation committee.

2.2 Experimental design

Fertile chicken eggs of Lohman Brown Classic strain [*Gallus gallus domesticus*] were randomly collected from a local chicken farm. Eggs were randomly assigned to three weight-matched groups and incubated for five days at 37°C with relative humidity of 60%, either untreated or after injecting 100 µl of sterilized sesame oil with 0.2 µg/ml [$^{2}H_{3}$]T or 0.58 µg/ml [$^{13}C_{3}$]A4 into the yolk, following the same procedure as in our previous study (von Engelhardt et al. 2009). These concentrations were within the physiological range (two times of the standard deviations) of the naturally present (unlabelled) T and A4 levels, which were determined using 200 mg homogenates of yolk plus albumen from unincubated eggs of the same batch. Although injection into the yolk at one particular spot may not adequately represent the distribution of maternal hormones over the different yolk layers, these layer structures quickly disappear after a few days of incubation. Moreover, most studies inject hormones dissolved in oil into the yolk as we did, so we can directly compare our findings with these studies. To determine the concentrations of labelled hormones, each treated egg was separated into three fractions: yolk-albumen, decapitated embryo (embryo heads were used for determination of steroid receptor expression as part of another study), and extra-embryonic membranes. Each fraction was homogenized separately and used for hormone extractions. Labelled hormones were extracted from 200 mg homogenates of yolk plus albumen of unincubated [day (d)0, n = 3) and incubated (d5, n = 3) eggs, individual decapitated embryos (E, n = 3), and 200 mg of extra-embryonic membranes (EM, n = 3) for each treatment. Because of small sample sizes (n = 3), no statistical tests were performed. To convert conjugated steroids to their free forms, single extracts were hydrolysed from 200 mg of yolk-albumen homogenate of incubated eggs, 100 mg of decapitated embryo homogenates, and 200 mg of extra-embryonic membranes for each treatment. To exclude the possibility that the lack of labelled androgens in the

decapitated embryonic tissues could be due to decapitation itself, T and A4 concentrations were determined using eggs from the same batch and the same analytical technique over the same time-period for intact embryos (head plus the rest of the body) using 100 or 300 mg homogenates.

2.3 Extraction of steroids

Each sample was extracted twice in 1 ml methanol by vortexing, followed by centrifugation at 12000xg for 10 minutes at room temperature. The supernatant was transferred to tubes containing 200 mg of solid $ZnCl_2$ for lipid precipitation (Wang et al. 2010). The total volume of the combined supernatants was made to 4 ml by adding 2 ml methanol, and centrifuged at 12000xg for 10 minutes at 4°C. The supernatant was dried under nitrogen gas in a waterbath at 50°C, re-suspended in 1 ml methanol, centrifuged at 12000xg for 10 minutes at room temperature, followed by addition of 1.8 ml water to the supernatant. This mixture was centrifuged at 12000xg for 10 minutes at 4°C. The supernatant was loaded on C18 SPE columns (3 ml, 500 mg, Grace Inc.) pre-equilibrated with 3 ml of methanol, followed by 3 ml of water. After collecting flow through, the column was washed with 3 ml water, and then eluted with 2 ml methanol. The average recovery for extraction was 85.1 ± 4.3% (mean ± s.d.) as measured for testosterone.

The conjugated steroid levels can be estimated as the difference between hydrolysed and non-hydrolysed extracts from the same samples as the hydrolysis converts a conjugated steroid into its free form (e.g. (Mi et al. 2014)). For hydrolysis of conjugates, the extract was dried under vacuum and re-suspended in 2 ml acetate buffer (0.5 M sodium acetate with 15 g/l sodium ascorbate, pH 4.8). 100 µl of commercial extract of *Helix Pomatia* (Suc d'Helix Pomatia, Brunschwig Chemie) was added, vortexed, and incubated at 46°C for 2 hours. The hydrolysed sample was cooled at room temperature and purified on HLB SPE columns (3ml, Waters Inc) pre-equilibrated with 2 ml of methanol, followed by 2 ml of water. After collecting flow through, the column was washed with 2 ml water, and then eluted with 2 ml methanol.

The eluent was dried under vacuum, re-suspended in 150 µl methanol, followed by addition of 350 µl water to make a final concentration of 30% methanol, and was analysed by mass spectrometry.

2.4 LC-MS/MS

The extracts were analysed by LC-MS/MS using the following two approaches. Firstly, HPLC was performed using a Shimadzu LC system, consisting of a SIL-20AC autosampler and two LC-20AD gradient pumps. Chromatographic separation was achieved at 30°C on a Alltima C18 column (2.1x150 mm 5 µm, Grace Davison Discovery Sciences). Eluent A was 100% H_2O and eluent B was 100% methanol without formic acid. The elution was performed starting at 10% B, followed by a linear gradient to 80% B in 2 minutes followed by a linear gradient to 95% B in 3 minutes. Then, the column was washed at 95% B for 7 minutes after which it was returned to the starting conditions. The flow rate was 0.25 mL/minute. The injection volume was 30 µL. The HPLC system was coupled to an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex). For positive ionization, the mass spectrometer was equipped with an atmospheric pressure chemical ionization (APCI) source. For negative ionization, a TurboIonSpray electrospray ionization (ESI) source was coupled to the mass spectrometer and a 1% ammonia solution in 50% methanol was introduced post-column with a flow rate of 0.05 mL/min. The source temperature was 450°C. Nitrogen was used as turbo heater gas, nebulizer gas and curtain gas. Tuning parameters were optimized for the selected reaction monitoring (SRM) ion pairs of the steroids for which standards were available. For the isotopically labelled compounds SRM ion pairs were calculated (see Supplementary Table S1). For each compound, two SRM ion pairs were determined, where the most intense was selected as quantifier while the other served as qualifier. For quantitation of the isotopically labelled compounds the standard addition method was applied using the non-labelled forms of the compounds. Data were collected and analysed with Analyst 1.5.2 software (Applied Biosystems/MDS Sciex).

In the second method, 25 µl of $^{13}C_3$ labelled 17-hydroxyprogesterone (30 nmol/L in 50% methanol, *IsoSciences*) was added as an internal standard and analysed with a XEVO TQ-S tandem mass spectrometer (Waters Corp.), equipped with an Online SPE Manager and ACQUITY UPLC system (Waters Corp.). The UPLC flow rate was set at 0.4 ml/min using 10 mM ammonium acetate, 0.1% formic acid in water and methanol (containing 0.1% formic acid) as mobile phases A and B respectively. For each extract, 40 µl sample was injected for extraction on a XBridge C8 cartridge and chromatographic separation was performed on a Kinetex C18 column (2.1 x 100 mm, 2.6 um). The mass spectrometer was operated under electrosprayionization mode with following operating conditions: cone voltage of 30 V, desolvation temperature of 600°C and source temperature of 150°C, collision energy of 15-40 eV optimized for different analytes. Quantitative calibration was performed by using a calibration curve using the internal standards for each of the analyte. The analysis was performed by monitoring two mass transitions for each analyte. The multiple reaction monitoring (MRM) transitions (m/z) are shown in the Supplementary Table S2. The quantification limit was 0.01 pg/mg of yolk or embryonic tissues for A4 and T; and 0.02 pg/mg for dihydrotestosterone (DHT).

3 RESULTS

Naturally occurring (unlabelled) T, 5α- or 5β-DHT, or their conjugated forms were not detectable in the intact chicken embryo (head plus rest of the body, excluding extra-embryonic membranes) after five days of egg incubation, and A4 was present at an extremely low concentration of 0.02 pg/mg. The levels of injected [$^{2}H_{3}$]T (Fig. 1A) and [$^{13}C_{3}$]A4 (Fig. 1B) declined strongly in the yolk-albumen homogenates during the first five days of incubation. Labelled T was no longer detectable after 5 days of incubation in the yolk-albumen homogenate, extra-embryonic membranes, or the embryo itself (Fig. 1A). Labelled A4 was present in only very low concentrations and only in the yolk-albumen homogenate after five days (Fig. 1B). Moreover, no labelled forms of neither DHT (one of the two biologically active metabolites of T), conjugated T or conjugated DHT were detectable in any egg fraction.

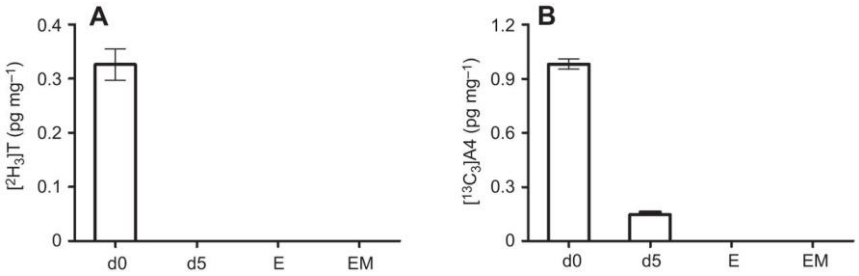

Figure 1. Concentration dynamics of injected stable isotope labelled [2H3]T and [13C3]A4 in fertilized chicken eggs. (A) [2H3]T and (B) [13C3]A4 concentrations assessed in yolk-albumen homogenates at day 0 (d0), day 5 (d5), in decapitated embryos (E) and in extra-embryonic membranes (EM). Values are means±s.e.m. of sample size of three per group. Labelled forms of DHT, conjugated T and conjugated DHT were not detectable in any fraction. Raw data are available in Table S3.

4 DISCUSSION

We found a lack of labelled T in all three egg fractions, and of labelled A4 in embryonic fractions, after five days of incubation. The use of internal standards and sample purification during extraction procedures using $ZnCl_2$ and solid phase extraction columns ensured that the lack of labelled compounds in embryonic fractions could not be due to matrix effect (affecting differences in ionization efficiency in the mass spectrometer among samples from different tissues). If there was an uptake of even 10% of the injected labelled hormones by the embryo, as suggested in previous studies based on the radioactivity transfer to embryonic chicken tissues (Benowitz-Fredericks & Hodge 2013; von Engelhardt et al. 2009), we would expect about 10 pg/mg [$^{2}H_{3}$]T and 50 pg/mg [$^{13}C_{3}$]A4 in the embryonic fractions

based on embryonic weight. However, after five days of incubation, no labelled T or A4 was found at all, despite quantification limits being as low as 0.01 pg/mg. This indicates that in the previous studies the labelled signal in embryonic tissue may either be due to conversion of injected androgens to other metabolites or may not have been from steroid hormones at all if the radioactive label detached from the hormone by metabolic processes.

Since the decline of androgen concentrations was found throughout the egg, it cannot be due to dilution with albumen or water, or embryonic uptake. This indicates that the maternal T and A4 are metabolized by embryonic and/or maternal enzymes, which is in line with the suggestions made in other studies (Gilbert et al. 2007; Kumar et al. 2018b; Paitz et al. 2011; Parsons 1970; Vassallo et al. 2014), as explained below. This raises the intriguing question as to which metabolic products these androgens are converted to, as this will determine the biological significance of maternal hormone deposition. Since the concentrations of both labelled T and A4 declined, interconversion of these two androgens cannot explain their decrease. Furthermore, neither of these steroids was converted to DHT, the androgen with the highest affinity for androgen receptor (Fang 2003) among the naturally occurring androgens, or the biologically inactive conjugated forms of T and DHT. Although our sample sizes are low (we were not aiming at quantitative differences between groups, but only at the verification of the uptake of injected yolk androgens by the embryo), such sample sizes have been used convincingly in other studies (Benowitz-Fredericks & Hodge 2013; von Engelhardt et al. 2009), and our results are very consistent within each group.

Although Benowitz-Fredericks and Hodge (2013) indicated conjugation of A4, A4 cannot be directly conjugated due to lack of a hydroxyl group, suggesting that it must have been conjugation of a metabolite of A4 in their study. It is noteworthy that DHT, which is often reported to be present in the yolk at day 0 in studies using radioimmunoassays (e.g. (Elf & Fivizzani 2002; Schwabl 1997a)), was not even detectable using mass spectrometry at day 0 or day 5, in spite of its very low limit of quantification (0.02 pg/mg), warranting some caution for the reliability of classical radioimmunoassays for hormone analyses of eggs.

Androgens are unlikely to be aromatized into estrogens (Fig. 2) at such an early stage of embryonic development as aromatase is expressed only after day 5 of incubation in chicken (Nomura et al. 1999), which is also experimentally supported by the lack of such early androgen conversion to estrogens (Parsons 1970). Parsons (1970) suggested 5β-androstane-3α, 17β-diol as the main metabolite in an *in vitro* study on metabolism of radiolabelled T by the two-day old chicken embryo; Paitz et al. (2011) suggested conjugated etiocholanolone as the main metabolite in the yolk-albumen homogenates of European starling eggs incubated for six days; and Kumar et al. (2018a) showed etiocholanolone and its conjugate as main androgenic metabolites in rock pigeon eggs incubated for 4.5 days.

These studies indicate a 5β-reduction, followed by 3α-hydroxylation metabolic route for maternal T and A4 (Fig. 2).

Considering the available information from above and based on the known metabolic pathways of functionally relevant androgens (Fig. 2), the following picture emerges: early in incubation, the maternal and/or embryonic enzymes are already metabolizing yolk T and A4 into 5β-androstane-3α, 17β-diol and/or etiocholanolone, and their conjugated forms.

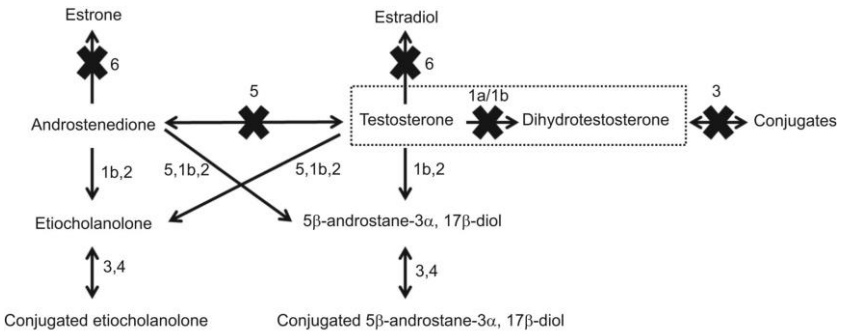

Figure 2. Suggested metabolic pathways for maternal T and A4 in the avian egg during early incubation. Double arrows represent reversible pathways and single arrows represent irreversible pathways, implying that the metabolites cannot be converted back to primary androgens during later stages of embryonic development. The pathways with interconversion between A4 and T, conversion of T into DHT, and conjugation of T and DHT were undetectable in the present study; whereas conversion to estrogens is very unlikely due to a lack of expression of the aromatase gene. All these non-detectable or improbable pathways are marked with a large cross. Key to steroidogenic enzymes: 1a, 5α-reductase; 1b, 5β-reductase; 2, 3α-hydroxysteroid dehydrogenase (HSD); 3, sulfotransferase/glucuronosyltransferase; 4, sulfatase/glucuronidase; 5, 17β-HSD; 6, aromatase.

The functional aspects of this metabolism are intriguing and create a paradox. Despite the well documented effects of avian yolk hormones on the embryo, chick and adult, the above-mentioned metabolites are thought to be biologically inactive. Both 5β-androstane-3α, 17β-diol and etiocholanolone are androgens with very weak binding affinity to the rat androgen receptor (Fang 2003). A high degree of sequence similarity in the ligand-binding domain of the mammalian and chicken androgen receptor (Katoh et al. 2006) suggests that these metabolites would not activate the chicken receptors. Conjugated androgens are thought to have no biological effect as they do not bind to androgen receptors but may rather be a way to inactivate the active forms. There are two ways for resolving this paradox. First, it has been argued that 5β-androstane-3α, 17β-diol and etiocholanolone may act independently of binding to androgen receptors and contribute to erythropoiesis (Paitz et al. 2011). This may explain the wide diversity of yolk androgen effects, as such a process may influence many developmental processes. Second, the conjugated metabolites may be de-conjugated back to their free forms of these metabolites in embryonic tissues, for which

some evidence was found for conjugated estrogen in turtle egg (Paitz & Bowden 2011). Both possibilities are open for experimental testing.

Recent studies on rock pigeon found no evidence for contribution of maternal yolk enzymes to early steroid metabolism (Kumar et al. 2018a,b), but perhaps there could be maternal enzymes in other egg components contributing to some extent to the early metabolism. Regardless of whether the enzymes are of maternal or embryonic origin, these could be an evolutionary adaptation to use maternal hormones prior to development of the embryonic endocrine systems at the optimal time and amount needed for the embryo itself, and/or to avoid detrimental effects of the relatively high yolk hormone concentrations (Groothuis & Schwabl 2002), as also suggested by Vassalo et al (Vassallo et al. 2014) for the stress hormone regulation in the later developmental stages. If the enzymes are of maternal origin, this would open the possibility that it reflects a maternal strategy by which the mother deposits different amounts of metabolizing enzymes depending on the environmental context. If the enzymes are of embryonic origin, it may reflect the evolutionary arms race between mother and offspring, as maternal hormone deposition may not always be advantageous to the chick (Groothuis et al. 2005b; Müller et al. 2007).

In conclusion, we have shown that when injected within a physiological range, the elevated yolk androgens (T and A4) are not taken up by the embryo but rather metabolized very early during incubation. It remains to be explored further whether the elevated androgen levels, before their depletion, could still exert any physiological effect on the embryonic development, for instance via receptor-mediated regulation of gene expression or by directly intercalating with DNA. If not, that would suggest that embryos have evolved the capacity to metabolize maternal hormones into biologically inactive or less potent metabolites, so that they can buffer their developing endocrine system against any detrimental consequences of such early exposure to maternal hormones, potentially playing a role in parent-offspring conflict. Alternatively, the metabolites themselves could be functional, a possibility that needs further exploration by simply injecting eggs with these metabolites and assessing their effects.

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SUPPLEMENTARY INFORMATION

Supplementary Table S1. SRM ion pairs and retention time of compounds measured by positive APCI. The first ion pair of each compound was used as quantifier and the second as qualifier.

Compound	SRM ion pairs (*m/z*)	Retention time (minutes)
A4	287.2/109.2 287.2/97.1	5.9
$^{13}C_3$-A4	290.2/112.1 290.2/100.1	5.9
T	289.2/109.2 289.2/97.1	6.06
$^{2}H_3$-T	292.3/97.2 292.3/109.2	6.06
$^{13}C_3$-T	292.2/112.1 292.2/100.1	6.06
DHT	291.2/255.1 291.2/273.1	6.46
$^{2}H_3$-DHT	294.2/258.1 294.2/276.1	6.46
$^{13}C_3$-DHT	294.2/258.1 294.1/276.1	6.46

m/z, mass-to-charge ratio; A4, androstenedione; T, testosterone; DHT, dihydrotestosterone.

Supplementary Table S2. MRM transitions monitored. The first ion pair of each compound was used as quantifier and the second as qualifier.

Compound	m/z	Cone voltage (V)	Collision energy (eV)
17-OH-P4	331/97	30	25
	331/109	30	28
$^{13}C_3$-17-OH-P4	334/100	30	25
	334/112	30	28
A4	287/97	30	35
	287/109	30	35
$^{13}C_3$-A4	290/100	30	35
	290/112	30	35
T	289/97	30	35
	289/109	30	35
$^{13}C_3$-T	292/100	30	35
	292/112	30	35
DHT	291/159	30	20
	291/255	30	15
$^{13}C_3$-DHT	294/162	30	20
	294/258	30	15

m/z, mass-to-charge ratio; V, volt; eV: electron-volt; 17-OH-P4, 17-hydroxyprogesterone; A4, androstenedione; T, testosterone; DHT, dihydrotestosterone.

Supplementary Table S3. Hormone concentrations (pg/mg) corresponding to Fig. 1.

	d0	d5	E	EM
[$^2H_3$]T	0.31	< 0.01	< 0.01	< 0.01
	0.29	< 0.01	< 0.01	< 0.01
	0.38	< 0.01	< 0.01	< 0.01
[$^{13}C_3$]A4	1.02	0.15	< 0.01	< 0.01
	0.93	0.17	< 0.01	< 0.01
	0.99	0.12	< 0.01	< 0.01